Universidade de Aveiro Departamento de Biologia
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Alterações Enzimáticas, Celulares e Histológicas em Peixes Expostos *in situ* ao Efluente de uma Mina

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia, realizada sob a orientação científica do Prof. Doutor Bruno André Fernandes de Jesus da Silva Nunes, Professor Auxiliar da Faculdade de Ciências da Saúde da Universidade Fernando Pessoa e da Co-orientação do Prof. Doutor Fernando José Mendes Gonçalves, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Professora Doutora Maria da Conceição Lopes Vieira Santos
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Professor Auxiliar com Agregação, Departamento de Ciências Exactas e Tecnológicas, Universidade Aberta

Professor Doutor Fernando José Mendes Gonçalves
Professor Auxiliar com Agregação, Departamento de Biologia, Universidade de Aveiro

Professor Doutor Bruno André Fernandes de Jesus da Silva Nunes
Professor Auxiliar, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa

Doutora Ruth Maria de Oliveira Pereira
Investigadora Auxiliar do CESAM, Universidade de Aveiro

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palavras-chave

efluente ácido da mina; *Carassius auratus*; biomarcadores; stresse oxidativo; micronúcleo; histopatologia; efeitos subletais

resumo

A presença de contaminantes nos meios aquáticos é atribuída a processos naturais, como por exemplo a lixiviação de solos, e a processos artificiais relacionados com os resíduos das actividades antropogénicas. Uma vez inseridos no sistema aquático, os contaminantes distribuem-se pelo material em suspensão, no sedimento, na água superficial e intersticial. A actividade mineira é um exemplo de uma fonte geradora de poluição no ambiente envolvente, sendo a descarga do efluente ácido da mina um dos mais graves problemas diagnosticados. A mina abandonada da Cunha Baixa (Mangualde, distrito de Viseu), pelo seu reconhecido impacto, serviu como objecto de estudo para o presente trabalho. Foram definidos como objectivos centrais (i) avaliar a toxicidade dos efluentes de três lagoas artificiais nas imediações da mina e mecanismos subjacentes em peixes, clarificando a relação entre a contaminação e os efeitos bioquímicos, citogenéticos e histopatológicos, e (ii) averiguar se as respostas ou efeitos biológicos obtidos podem constituir indicadores (biomarcadores) de efeito a contaminantes.

O presente trabalho pretendeu desenvolver um ensaio ecotoxicológico *in situ* em indivíduos de *Carassius auratus,* alicerçado na quantificação de parâmetros subletais. Foram desenvolvidas câmaras de ensaio adequadas para expor organismos ao efluente das três lagoas artificiais em estudo. Os animais foram expostos (i) ao efluente da lagoa de referência (Ref), (ii) ao efluente ácido da mina (M), que se acumula numa lagoa subterrânea, (iii) e ao efluente da lagoa de tratamento (T), onde se procede à neutralização do supra-mencionado efluente. Os períodos de exposição foram 8, 16, 24 e 48h. Após cada período, os animais foram sacrificados para se proceder à quantificação de vários biomarcadores enzimáticos, de genotoxicidade e histopatológicos.

Na primeira parte do trabalho, referente à abordagem de nível bioquímico/metabólico, foram estudadas as respostas de stresse oxidativo e do metabolismo de destoxificação do fígado, a partir da análise das enzimas catalase (CAT) e glutationa-S-transferases (GSTs), respectivamente. No músculo dorsal, a actividade da enzima lactato-desidrogenase (LDH), um biomarcador respiratório, forneceu informações sobre o estado metabólico dos organismos. Foram investigadas, ainda, as respostas de neurotoxicidade, a partir análise da enzima acetilcolinesterase (AChE) no tecido nervoso.

Na segunda parte do trabalho foi avaliada, ao nível citogenético, a acção genotóxica da exposição à radioactividade e ao stresse químico, por intermédio do teste da frequência de micronúcleos (MN) e de outras anomalias nucleares eritrocíticas (ENA) no sangue periférico e da observação das alterações histopatológicas ao nível do fígado.

Todos os peixes expostos ao efluente M morreram durante as primeiras 8h de exposição. Relativamente à exposição aos efluentes Ref e T, os animais não apresentaram alterações significativas nos parâmetros de stresse oxidativo e de neurotoxicidade. Pelo contrário, os parâmetros associados aos metabolismos de destoxificação e de respiração anaeróbia evidenciaram diferenças significativas pontuais, respectivamente, na actividade das enzimas GSTs e LDH, quando comparados com os mesmos tempos de exposição em ambos os efluentes.

Na avaliação da genotoxicidade, os peixes expostos ao efluente Ref apresentaram um ligeiro aumento da frequência de MN, embora não significativo. No teste de ENA, observou-se a indução das anomalias ao longo do tempo, embora não se tenham observado diferenças na sua frequência entre os locais em estudo. Ao nível histopatológico, os peixes expostos aos efluentes Ref e T não expressaram alterações morfológicas significativas ao nível do fígado.

A estratégia adoptada, baseada na análise integrada de diferentes indicadores, incluindo os níveis molecular (respostas fisiológicas de stresse, como as alterações enzimáticas), subcelular/celular (lesões genéticas como o aparecimento de micronúcleos), tecidos e órgãos (alterações histopatológicas), aplicados ao ciprinídeo *C. auratus*, demonstrou a sua aplicabilidade na monitorização ambiental de contaminação por metais e/ou misturas complexas resultantes do extracção de minério.

Keywords acid mine drainage, *Carassius auratus*, biomarkers, oxidative stresse, micronuclei, histopathology, sublethal effects

Abstract

The occurrence of contaminants in the aquatic environment is due to natural processes, such as soil runoff, but it can also be attributed to artificial processes related with anthropogenic activities and their resulting wastes. Once in the aquatic system, the contaminants may be associated to the suspended matter or distributed along the sediment, surface and interstitial waters. In particular, mining activities are one of the sources of environmental pollution in nearby areas, where the release of acidic effluents represents a serious utmost problem. According to this trend, the abandoned uranium mine of Cunha Baixa (Mangualde, Viseu), due to its recognised impacts, was defined as the study area of the present work. Overall, the main goals addressed in this work concerned (i) the toxicity assessment of effluents coming from three artificial lagoons in the vicinity of the mine area and the underlying mechanisms that threat fish species, in order to clarify the relationship between contamination and biochemical, citogenetic and histopathological effects; and (ii) the discussion about the potential use of biological responses or effects as effect indicators (biomarkers) concerning the presence of contaminants. In this way, ecotoxicological *in situ* assays were performed with *Carassius auratus*, for subsequent quantification of sublethal endpoints. Thereby, appropriate *in situ* chambers were developed for the exposure of organisms to the effluent from three artificial lagoons: (i) effluent from the reference lagoon (Ref), (ii) acid mine effluent (M) accumulated in a subterranean lagoon, (iii) effluent from the treated lagoon (T), where the acid effluent was neutralized. The exposure periods were 8, 16, 24 and 48h. After each period, animals were sacrificed for the quantification of several enzymatic, genotoxic and histopathological biomarkers.

Along the first working part, which was related to the biochemical/metabolic approach, we analysed responses of oxidative stress and of hepatic detoxification metabolism, through the determination of catalase (CAT) and glutathione-S-transferases (GSTs) activities, respectively. In the dorsal muscle, the activity of lactate dehydrogenase (LDH), a respiratory biomarker, provided information about the organisms´ metabolic state. Moreover, the neurotoxic responses were evaluated through the measurement of acetylcholinesterase (AChE) activity, in the nervous tissue.

On the other hand, in the second part of this work, we assessed the genotoxic potential of radioactivity and chemical stresses at a citogenetic level, by performing the tests, in the peripheral blood, of the micronucleus (MN) and other erythrocytic nuclear abnormalities (ENA), and by observing histopathological changes in the liver.

The obtained outcome evidenced that all fish exposed to M effluent died during an 8h-period. In which concerns the exposures to Ref and T effluents, the animals did not reveal significant changes on the oxidative stress and neurotoxicity parameters. In contrast, the parameters used for the measurement of detoxification metabolism (GSTs activity) and the anaerobic respiration (LDH activity) depicted discrete significant differences, when compared with the same exposure periods, for both effluents.

In what concerns the genotoxicity evaluation, fish exposed to the Ref effluent showed a slight, though not significant, increase in the MN frequency. The test of ENA revealed an enhancement of those abnormalities along with an increase of exposure periods, albeit no differences were observed in their frequency between the study sites. The liver of fish exposed in both ponds did not reveal significant histopathological changes in their structure.

The adopted strategy, based on an integrated analysis of different indicators at a molecular (physiological responses of stress, like enzymatic changes), subcelular/cellular (genetic damages, such as the occurrence of micronuclei), and tissue and organs (histopathological alterations) levels, on the cyprinid *C. auratus*, proved to be an useful tool for monitoring the environmental contamination induced by metals and/or complex mixtures resulting from ore extraction.

Da determinação que tens tomada,
Não tornes por detrás, pois é fraqueza
Desistir-se da cousa começada

Luís Vaz de Camões *in* Os Lusíadas

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CAPÍTULO I

Introdução Geral

Introdução Geral

1. Metais – fonte de contaminação do ambiente

A actividade humana exerce uma influência significativa nos ciclos da matéria e da energia no ambiente natural, pelo que se reveste de particular interesse estudar as interferências e influências dos elementos químicos considerados tóxicos, particularmente quando estão presentes em concentrações anómalas (Oliveira, 1997). Assim, da prática de determinadas actividades (e.g. agricultura e indústria), quando exercidas de forma descontrolada e sem um devido acompanhamento técnico e tecnológico, podem gerar-se ou libertar-se elementos e compostos perniciosos, sob várias formas.

As actividades de exploração mineira, quer se desenvolvam a céu aberto, quer em lavra subterrânea, podem também gerar situações e/ou efeitos de potencial perigosidade para o meio envolvente, devido ao impacto, em particular de natureza química, que daí pode resultar (Oliveira e Ávila, 1995). Estudos recentes vieram demonstrar que a expressão desta actividade pode ocasionar danos diferenciados, quer ao nível da contaminação dos recursos hídricos (e.g. Marín-Guirao et al., 2007; Pandey et al., 2007), dos solos (e.g. Antunes et al., 2008; Jian-Min et al., 2007; Jung, 2001) e da atmosfera (e.g. Wang et al., 2007b; Chaulya, 2004), quer ainda no que respeita à intensidade do impacto da radioactividade (Carvalho et al., 2007), proveniente da natureza dos minérios explorados.

As minas de urânio, em particular, produzem uma quantidade significativa de resíduos com influência potencialmente negativa, que resulta do material radioactivo (i.e. urânio e radionuclídeos descendentes do urânio) e da elevada concentração de metais (e.g. Pb, Mn, Al, Cu, Cd, Zn) (Pandey et al., 2007; Burke e Banwart, 2002), sobretudo no caso das minas abandonadas (Lozano et al., 2000, 2002). De salientar também, que os seus resíduos se caracterizam por um baixo pH, o que aumenta o poder de dissolução e transporte dos elementos químicos tóxicos, até distâncias consideráveis da origem (Oliveira, 1997). Frequentemente, os depósitos resultantes desta actividade são ricos em sulfuretos, como por exemplo a pirite, e a sua oxidação origina a drenagem ácida da mina, passando esta a apresentar elevados teores de mineralização (Ferreira da Silva et al., 1995). Tiwary et al. (2001) salientam que este fenómeno de drenagem ácida está relacionado com a reacção destes minerais com a água e oxigénio na presença da bactéria *Thiobacillus*, responsável pela produção de ácido sulfúrico e hidróxido/sulfato de ferro.

Em Portugal, a existência de jazigos urano-radíferos no subsolo, exploráveis em condições económicas, permitiu que se criasse, desde o princípio do século XX, uma indústria para a produção de concentrados de rádio e, mais tarde, de urânio. Carvalho et al. (2007) mencionam que aproximadamente 60 locais foram explorados, na sua maioria situados nos distritos da Guarda e Viseu. A Mina da Cunha Baixa foi um desses locais. Esta mina localiza-se na periferia nordeste da povoação de Cunha Baixa (Mangualde), a 20 km de Viseu, e integra-se na região uranífera das Beiras (Oliveira e Ávila, 1998). Esteve em laboração desde 1967 até 1993, ano em que cessou a sua actividade (Oliveira e Ávila, 2001). As características do jazigo em apreço possibilitaram a exploração subterrânea e a céu aberto, sendo que os minérios extramarginais eram tratados em eiras de lixiviação estática (Portugal e Ferreira, 1971), *in situ*, com ácido sulfúrico (H_2SO_4) (Oliveira e Ávila, 2001). Desta prática eram recolhidos os licores de lixiviação, sendo posteriormente o minério rico transportado para o complexo principal – a mina da Urgeiriça – onde se procedia ao seu tratamento e subsequente industrialização (Cordeiro Santo e Pereira Freire, 1983).

Nos últimos anos de exploração da mina (1984 a 1991), era comum recorrer-se à lixiviação de minério, com o intuito de se recuperar uma maior quantidade de minério viável (Antunes, 2007). Os depósitos daí resultantes continham sulfuretos – minerais particularmente instáveis nas condições terrestres – cuja oxidação origina uma drenagem ácida do efluente da mina (Ferreira da Silva et al., 1995). Este facto, associado à presença de escombreiras no local, potenciou a contaminação dos sistemas aquáticos, a partir da lixiviação e simultânea incorporação dos metais nos solos circundantes, atingindo assim os lençóis freáticos.

Em 1993, sem que tivesse ocorrido uma intervenção que evitasse consequências ambientais, esta mina foi entregue ao abandono. Antunes (2007) menciona que após o encerramento da mina, a empresa que deteve a sua concessão (Empresa Nacional de Urânio, ENU) implementou algumas medidas de recuperação ambiental para a área envolvente à mesma. Neste sentido, criou-se uma estação de tratamento de águas – neste estudo referenciada como lagoa de tratamento – cujos objectivos são a neutralização do efluente aquático ácido (resultante da lavagem do minério com H_2SO_4) com hidróxido, e a posterior precipitação utilizando sulfato de bário (Carvalho et al., 2007). Este composto permite induzir a precipitação de compostos de rádio, assim como a remoção de metais, e o aumento simultâneo do valor de pH para a zona neutra (≈ 7). Do tratamento do efluente ácido, passaram a acumular-se lamas residuais na base da lagoa que, embora menos radioactivas, contêm elevadas concentrações de radionuclídeos de urânio (Carvalho et al., 2007) e de outras espécies metálicas, assim

como uma mistura complexa de metais. Uma vez que estas lamas e/ou precipitados eram retirados da lagoa e colocados nas suas imediações da mesma, actualmente é possível observá-los a céu aberto. Esta prática, vista enquanto recobro do efluente ácido, tornou-se o âmago do nosso estudo, na medida em que nos propusemos averiguar e/ou avaliar, do ponto de vista ecotoxicológico, os potenciais perigos implicados neste local, nomeadamente os riscos associados à exposição ambiental dos produtos de neutralização do efluente, sobretudo quando os seus produtos de lixiviação alcançam o compartimento aquático circundante à mina.

Antunes et al. (2007a, 2007b), Freire Ávila et al. (2005) e Pereira et al. (2005) desenvolveram estudos que explicitam os perniciosos efeitos ambientais associados ao abandono de minas em Portugal. Nos últimos anos, deu-se início ao desenvolvimento de projectos de carácter técnico e de investigação que permitiu abreviar a tomada de medidas propensas, designadamente, ao diagnóstico de situações de potencial perigosidade e a aplicação de soluções de controlo, contenção e/ou reabilitação ambiental. Este esforço foi igualmente acompanhado por uma reestruturação da legislação, tomando proporções transparentes de maior rigor e exigência ambientais.

2. Peixes – organismos de teste em toxicologia aquática

Os sistemas aquáticos são entendidos, sem paradoxos, como os principais receptores de inúmeros poluentes, pois para além das descargas directas, são ainda alvo da contaminação resultante da deposição atmosférica e lixiviação do solo (Pacheco, 1999). A bioacumulação de contaminantes nos tecidos e órgãos de organismos aquáticos tem sido, por todo o mundo, desígnio de estudo intenso, tendo-se adoptado a concepção de que são excelentes indicadores da avaliação e monitorização da qualidade ambiental (e.g. Yarsan et al, 2007; Henry et al., 2004). Estudos recentes (e.g. Alibabić et al., 2007; Smith et al., 2006; Papagiannis et al., 2004; Rashed et al., 2001) demonstram que os peixes são dos organismos mais sensíveis e que mais indicações nos concedem relativamente à presença de substâncias tóxicas na água, estando particularmente bem documentada a toxicidade provocada pelos metais (e.g. Delistraty et al., 2007; Fernandes et al., 2007; Petrlova et al., 2007). Rayment e Barry (2000) explicam esta preferência ao remeter para a acessibilidade destes organismos (quer na natureza em estado selvagem, quer em aquacultura) e na simples, rápida e económica preparação e análise das amostras, relativamente aos demais métodos alternativos comummente utilizados na investigação da toxicidade em água e sedimentos. No que respeita às suas características fisiológicas, os peixes têm a capacidade de desencadear mecanismos

adaptativos que se baseiam na manutenção do equilíbrio do meio interno – dependentes de um controlo homeostático manifestamente coordenado – que lhes permite sobreviver, crescer e reproduzir (Pacheco, 1999). Este autor refere, ainda, que esta adaptabilidade assenta numa complexa rede de respostas, como as alterações hormonais, fisiológicas ou metabólicas, que podem constituir excelentes indicadores da interacção do organismos com o(s) agente(s) químico(s) que, directa ou indirectamente, afectam a qualidade ambiental.

Foi já levado a cabo um número considerável de estudos em peixes da espécie *Carassius auratus*, dedicados a aspectos como as respostas a agentes tóxicos e de stresse (e.g. Mimeault et al., 2006; Sharifi et al., 1997), os mecanismos de recuperação de alterações nutricionais (e.g. Volkoff et al., 2001; Bandyopadhyay et al., 2005; Kestemont, 1995), ou ainda, alterações citogenéticas (e.g. Wang et al., 2007a; Luo et al., 2006). Estes estudos constituem, portanto, uma base útil de trabalho, dado proporcionarem um conhecimento prévio da fisiologia e do comportamento da espécie.

3. Biomarcadores - metodologia em uso

Os seres vivos possuem uma capacidade intrínseca de resistir à agressão, incluindo a do tipo químico, e que é mediada por uma série de alterações fisiológicas que tendem a manter a homeostasia. No entanto, esta resposta adaptativa é limitada. É na sequência das alterações fisiológicas que representam a sua capacidade de adaptação, ou já para além dela, que os organismos podem exibir alterações que se consideram efeitos tóxicos. São estas as respostas de stresse à exposição aos agentes tóxicos, entre os quais se destacam os metais. Pela sua elevada toxicidade e pelo facto de não serem degradados pela natureza, a maioria dos metais assume considerável importância, na medida em que a sua incidência e persistência no ambiente pode provocar, a longo prazo, danos irreparáveis (Benassi et al., 2006).

Assim, a utilização de biomarcadores, quer para monitorização da qualidade ambiental, quer para a obtenção de informação relativa à condição dos organismos que se encontram em ecossistemas poluídos, tem recebido crescente atenção nos últimos anos (de la Torre et al., 2007; Ozmen et al., 2006; Lionetto et al., 2003; Lopes et al., 2001).

A definição de biomarcador tem sido alvo de alguma polémica. Todavia, de um modo mais ou menos consensual, podemos defini-lo como alterações bioquímicas, histológicas ou fisiológicas induzidas por contaminantes e que podem ser mensuráveis num sistema ou amostra biológica (Timbrell, 1998). Os biomarcadores podem ser usados

para várias finalidades, dependendo do objectivo do estudo, do tipo de exposição química e da interacção da substância química com os receptores biológicos. Mais especificamente, do ponto de vista bioquímico, podemos falar de biomarcadores de neurotoxidade (e.g. actividade da enzima acetilcolinesterase), por reflectirem quaisquer efeitos adversos numa estrutura ou função dos sistemas nervoso central e/ou periférico, por força de agente biológico, químico ou físico (Slikker et al., 2005); biomarcadores de proliferação peroxissomal/stresse oxidativo (e.g. actividade de enzima catalase), cuja função fisiológica é a de decompor o peróxido de hidrogénio – um prejudicial agente oxidante – em água e oxigénio, que está relacionado com diversas patologias ligadas ao stresse oxidativo; biomarcadores de metabolismo de destoxificação por conjugação com glutationa nos processos de fase II (e.g. actividade das glutationa-S-transferases), responsáveis pela biotransformação de substâncias potencialmente nocivas, em substâncias de elevada solubilidade em água, e regra geral, mais baixa toxicidade; biomarcadores respiratórios (e.g. a actividade da enzima lactato-desidrogenase), cuja actividade permite avaliar o estado metabólico dos organismos, no que diz respeito ao predomínio do metabolismo anaeróbio sobre o aeróbio.

Os biomarcadores de genotoxicidade permitem averiguar quanto à integridade do material genético. Contudo, esta condição essencial à sobrevivência das células e dos organismos é susceptível de ser alterada pela exposição a contaminantes ambientais (Pacheco, 1999). A contagem de micronúcleos (MN) é um método amplamente utilizado na avaliação do impacto biológico da poluição aquática, testando, assim, a genotoxidade de determinados compostos, após uma exposição *in vivo* (Çavaş e Ergene-Gözükara, 2005; Sanchez-Galan et al., 2001). O teste de MN, por permitir a detecção de perdas e/ou quebras de cromossomas durante a mitose, de um modo simples, rápido e eficaz, é um dos métodos preferidos na avaliação de danos genotóxicos (Fenech, 2000). Para além deste facto, o teste de MN em eritrócitos de peixes têm sido aplicado na avaliação do impacto genotóxico de poluentes ambientais, tendo revelado, quer em ensaios laboratoriais quer no campo, uma interessante correlação com a exposição a vários agentes químicos e físicos (Gustavino et al., 2001; Al-Sabti et al., 1995). Foi, entretanto, sugerido que a detecção de MN em eritrócitos de peixes poderia ser complementada com a quantificação de outras anomalias nucleares eritrocíticas (ANE) (Carrasco et al., 1990). Actualmente, muitos autores (e.g. Guilherme et al., 2008; Pacheco e Santos, 2002) assumem que os testes de ANE e de MN indiciam a presença de dano genético.

As alterações nos órgãos, ou sistemas de órgãos, constituem respostas a níveis organizacionais mais elevados, que reflectem danos prévios e citotoxicidade (Pacheco, 1999). Neste sentido, os estudos histopatológicos são recomendados como suplemento

de estudos bioquímicos, permitindo consolidar o entendimento das relações causa-efeito entre exposição e respostas a níveis organizacionais mais baixos (Schwaiger et al., 1997). A sua aplicabilidade mostrou ser extensível a vários tipos de contaminantes, como metais (e.g. Barni et al., 2007; Pereira et al., 2005; Gül et al., 2004), hidrocarbonetos policíclicos aromáticos (e.g. Nero et al., 2006; Pietrapiana et al., 2002), ou efluentes industriais (e.g. Pacheco e Santos, 2002).

Deste modo, os biomarcadores apresentam múltiplas vantagens fundamentais: um biomarcador deve ser mensurável antes de se sentirem efeitos adversos a níveis mais elevados de complexidade; os métodos para a sua quantificação devem ser simples, rápidos e de baixo custo; deve ser específico para um ou para uma classe de contaminantes; ser passível de ser aplicado a um número abrangente de espécies (Newman, 1998). Contudo, para que se verifiquem estas e outras vantagens, é necessário que na sua escolha sejam tidas em consideração as características do organismo, assim como as condições ambientais em teste, a resposta esperada, o(s) tóxico(s) a analisar, a via de exposição, a duração da exposição em função dos tóxicos e respectivas concentrações.

4. Objectivos da dissertação

A zona da Cunha Baixa (Mangualde, distrito de Viseu), por ainda hoje reflectir a intensa actividade extractiva que se prolongou por mais de duas décadas, é uma das regiões mais afectadas por este problema em Portugal Continental. Esta mina de urânio, durante a sua actividade, emitiu para o meio envolvente descargas contínuas de efluente ácido, originando a dispersão e acumulação de uma mistura complexa de elementos químicos e reactivos no ambiente. Para além de outros, os fenómenos de erosão e/ou lixiviação, promoveram a transferência destes elementos pelos vários compartimentos ambientais (Pedrosa e Martins, 1999; Oliveira e Ávila, 1998). Actualmente, na zona envolvente à mina, verifica-se que da exploração resultou um sistema aquático composto por três lagoas artificiais (Antunes et al., 2007a,b): uma acumula água da chuva e/ou do aquífero (onde, por um curto período de tempo, foi feita a extracção a céu aberto) (mencionada como lagoa de referência [Ref] ao longo do presente trabalho); uma outra lagoa que se encontra junto da zona de exploração da mina, que corresponde ao afloramento do aquífero que percorre as galerias e, por isso, apresenta baixo pH (a que se designou “buraco da mina” [M]); e uma lagoa que recebe o efluente bombeado da lagoa [M] e onde é neutralizado e precipitado (lagoa de tratamento [T]).

Em consequência do seu reconhecido impacto, esta mina integra desde 2003 o Programa de Reabilitação das Áreas de Minas Abandonadas. Contudo, apesar da implementação de técnicas de reabilitação ambiental, coloca-se a necessidade de avaliar, em particular, o estado da qualidade das águas superficiais, averiguando o seu impacto em organismos aquáticos, como peixes.

Assim, o presente trabalho desenvolveu-se nas três lagoas, onde se procedeu à exposição *in situ* de peixes (*Carassius auratus*), por já se ter revelado um excelente indicador da qualidade dos ambientes aquáticos (Sun et al., 2007; Zhang et al., 2005).

Os objectivos centrais deste estudo consistiram em:

- avaliar os efeitos induzidos pela exposição aos metais existentes nos efluentes de uma mina de urânio (antes e após tratamento) e à radioactividade, por intermédio da quantificação das enzimas acetilcolinesterase (AChE), catalase (CAT), glutationa-S-transferases (GSTs) e lactato-desidrogenase (LDH), em peixes da espécie *Carassius auratus*. Estas enzimas foram seleccionadas como presumíveis biomarcadores de efeito ao nível de disrupção de neurotransmissão, de proliferação peroxissomal/stresse oxidativo, de destoxificação e de metabolismo anaeróbio, respectivamente;

- estudar as alterações celulares e histopatológicas em hepatócitos e o desenvolvimento de MN e de outras anomalias nucleares eritrocíticas (indicadores de dano genético), como biomarcadores de efeito, induzidos pela exposição aos efluentes de uma mina de urânio em peixes (*Carassius auratus)*;

- averiguar se as respostas ou efeitos biológicos obtidos, tanto ao nível dos biomarcadores bioquímicos, genotóxicos e histopatológicos, podem constituir indicadores (biomarcadores) de efeito a contaminantes, como metais existentes num efluente de uma mina de urânio.

CAPÍTULO II

The effectiveness of a chemical treatment to mitigate the toxicity of uranium mine drainages to *Carassius auratus*

Bessa, M.L., Pereira, R., Antunes, S.C., Gonçalves, F., Nunes, B.

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The effectiveness of a chemical treatment to mitigate the toxicity of uranium mine drainages to *Carassius auratus*

ABSTRACT

The production of an acidic mine effluent rich in a mixture of metals (e.g. U and Sr) and radionuclides, is the main legacy left by uranium mines. A chemical treatment with hydroxide and barium sulphate is usually applied by mining companies to deal with the problem. The effectiveness of such treatment is only assessed comparing the final chemical properties and the concentrations of metals with those legally established. In this study, freshwater fishes (*Carassius auratus*) were exposed *in situ* (8, 16, 24, and 48 h) within three ponds from the Cunha Baixa uranium mine (Portugal): a reference pond (Ref), the mine pit pond (M) and a treatment pond (T), which receives the treated effluent. Several biomarkers were evaluated, at each exposure period, namely: i) acetylcholinesterase activity (AChE); ii) catalase activity (CAT); iii) glutathione-S-transferases activity (GSTs); iv) lactate dehydrogenase activity (LDH); v) erythrocytic micronucleus (MN) frequency and other erythrocytic nuclear abnormalities (ENA) and, vi) liver histopathological alterations. The chemical treatment was effective in terms of pH neutralization. Nevertheless some metals persist at higher concentrations, potentially contributing for the induction of oxidative stress or increased conjugation activity in fish. GSTs and LDH were the most sensitive biomarkers within the timeframe of the *in situ* assay performed.

1. INTRODUCTION

Uranium (U) is a radioactive element of natural occurrence in the environment, and is outstanding among the global environmental pollutants, since it is released into the environment by mining activities. Within these areas, concerns with the aquatic resources are particularly meaningful when *in situ* leaching of poor ore, with sulphuric acid is performed, which is a common procedure to extract poor ore during the final phases of extraction. The generic Portuguese scenario shows that these leaching activities were also adopted, and gave rise to acidic mine effluents rich in uranium, strontium, radium, radionuclides, stable metals (Zn, Mn, Fe, Cu, Cd, Pb, Ni, Co, etc.), arsenic and sulphates.

However, the mentioned species were not the only ones, since other metallic compounds may be present at the same time in the treatment effluent (Carvalho et al., 2007). In order to avoid deleterious environmental consequences, it is common practice to employ a chemical treatment, involving the neutralization and the precipitation of radionuclides and metals with hydroxide and barium sulphate, respectively. The sludge produced within the treatment plant, contaminated with elevated radioactivity levels and high concentrations of metallic species, contributes to the contamination of the terrestrial and aquatic compartments, when removed and spread locally. Hence, the high environmental and health risks of mining areas are associated with a mixture of complex elements exceeding water, soil and sediment guideline values (e.g. Pereira et al., 2008) and with ecotoxicological impacts on aquatic (Antunes et al., 2007a-b) and terrestrial species (Antunes et al., 2008; Antunes et al., *in press*).

The toxic effects of metals usually involve interactions between inorganic forms of metals and cellular targets, which can be specific enzymes, proteins of transport and receptors on cells and organelle membranes. Such interactions may be responsible for increased the bioaccumulation, may also be elicited through the inhibition of excretion, but can contribute for the onset of defense mechanisms such as overexpression of metallothioneins (Manahan, 2003; Goyer and Clarkson, 2001) Some tissue specificity occurs in metal binding; therefore, metals tend to accumulate in target organs, yielding toxic effects when their concentration surpasses a threshold level (Manahan, 2003).

Several freshwater fish species have been considered good test organisms, indicators of contamination by anthropogenic pollutants (e.g. Ergene et al., 2007; Ozmen et al., 2006; Papagiannis et al., 2004). Hence, the evaluation of a battery of biomarkers on fish species became a useful routine in Ecotoxicology, to assess the effects of environmental chemical contamination (Nunes et al., 2006). Acetylcholinesterase (AChE) plays a central role in the mechanism of neurotransmission, since it catalyses the cleavage of acetylcholine in choline and acetic acid, after its release at the nervous cleft of cholinergic synapses. In the central nervous system, this enzyme plays a role in the function of the peripheral neuromuscle junctions. Inhibitors of acetylcholinesterase – e.g. several pesticides and metals - affect certain nerve junctions in animals, as well as parasympathetic effector sites (Leblanc, 2004). The transmission of impulses across nerve junctions involves the release of a transmitter chemical, which, in the case of many nerves, is acetylcholine. To stop transmission, the neurotransmitter acetylcholine, must be broken down immediately after it has made is function. AChE-inhibiting neurotoxic compounds can cause serious dysfunction in aquatic organisms, e.g., behavioral changes, paralysis and death (Fulton and Key, 2001). The enzyme catalase (CAT) has

been used as an indicator parameter of peroxisome proliferation, since it is frequently present in the mentioned subcellular structure and as an oxidative stress biomarker (Schrader and Fahimi, 2006). Its physiological role is also of great importance, since it converts hydrogen peroxide, a powerful and potentially harmful oxidizing agent, into oxygen and water. It also uses hydrogen peroxide to oxidize toxins, transforming them into innocuous compounds, such as water (H_2O) and molecular oxygen (O_2) (Van der Oost et al., 2003). Its use as an indicator of oxidative stress, due to the fact that it is one of the main intermediate species in oxidative scenarios, using hydrogen peroxide derived from the dismutation of the superoxide radical by superoxide dismutase (Hodgson and Levi, 2004). The overexpression of CAT by the nucleus is thus a response to oxidative insult, and CAT may serve as a reliable indicator of oxidative stress induced by chemicals (e.g. Atli et al., 2006; Sanchez et al., 2005; Gül et al., 2004). Glutathione-S-transferases (GSTs) play an important role in the biotransformation and detoxification of a number of electropholic compounds, by conjugation with reduced glutathione (Rao, 2006), forming substances of low toxicity/high water solubility. Regarding the effect of metals and other contaminants on this group of enzymes, various mechanism of action and contradictory effects have been reported: some authors reported their induction (e.g. Ahmad et al., 2000; Canesi et al., 1999; Regoli and Principato, 1995), while other showed evidences of inhibition (e.g. Sen and Semiz, 2007; Gravato et al, 2005). Lactate dehydrogenase (LDH) is also an important enzyme in biological systems, which is induced by oxygen stress (Wu and Lam, 1997). It is responsible for catalyzing the conversion of lactate to piruvate – an essential step in producing cellular energy - with concomitant conversion of NADH to NAD^+. Its activity in muscle or in whole body homogenates has been used as indicative of potential effects on energy production mechanisms induced by chemical stress (Frasco and Guilhermino, 2002; De Coen et al., 2001).

Genetic biomarkers have been used in different aquatic organisms such as molluscs (e.g., Bolognesi et al., 2006; Magni et al., 2006), amphibians (e.g., Barni et al., 2007; Lajmanovich et al., 2006) and fish (e.g., Baršienė et al., 2006; Matsumoto et al., 2006; Al-Sabti and Metcalfe, 1995) to detect the effects of environmental contamination. The micronucleus (MN) test has been considered one of the most useful methods for evaluating genotoxicity in aquatic systems and has been extensively applied on fish species (e.g. Lemos et al., 2007; Udroiu , 2006; Çavaş and Ergene-Gözükara, 2005; Al-Sabti and Metcalfe, 1995). MN are produced from chromosome fragments or whole chromosomes that lag at cell division due to the lack or damage of a centromere, or an abnormal cytokinesis (Baršienė et al., 2006). The evaluation of MN occurrence in peripheral blood is a widely used method to look for clastogenic and aneugenic effects

(e.g. Stoiber et al., 2004; Nesslany and Marzin, 1999; Sanches-Galan et al., 1998). Moreover, MN in fish erytrocytes have revealed interesting correlations with the exposure to a number of chemical and physical agents (Gustavino et al., 2001; Al-Sabti and Metcalfe, 1995). Besides MN frequency, other authors (Marques et al., *in press*; Serrano-Garcia and Montero-Montoya, 2001; Ayllón and Garcia-Vasquez, 2000) have also considered other markers for the evaluation of similar damage, such as the simultaneous expression of morphological erythrocytic nuclear abnormalities (ENA). These nuclear abnormalities have been described by Carrasco et al. (1990), and interpreted as nuclear lesions analogous to MN (Ayllón and Garcia Vazquez, 2001; Pacheco and Santos, 1996). Due to its higher responsiveness, ENA assay represents an alternative to MN test overcoming a possible lack of sensitivity related to the low frequency of MN in some fish species.

At a higher level of biological organization, histological changes in animal tissues have proved to be a sensitive biomarker to assess exposure to metals and, subsequent effects, under laboratorial and environmental conditions, both to aquatic (e.g. Tkatcheva et al., 2004; Arellano et al., 1999) and terrestrial species (e.g. Pereira et al., 2006; Odendaal and Reinecke, 2003). The liver, in particularly, is susceptible to damages yielded by a variety of toxicants (Schlacher et al., 2007), since it is the main organ involved in detoxification mechanisms.

Hence, the main objective of the present study, integrated in the ecological risk assessment of the Cunha Baixa uranium mine (Center of Portugal), was to assess the effectiveness of the chemical treatment applied to the acidic effluent that stills being produced, in terms of reduction of its ecotoxicological hazard to aquatic species. A vertebrate key species from aquatic food-chains, a fish species (*Carassius auratus*) was chosen for the *in situ* exposures in the mine ponds, and the enzymes AChE, CAT, GSTs, and LDH, were selected as endpoints for the assessment of sub-lethal effects after exposure to effluents from a uranium mine. These enzymes have a key role in biological processes determinant for the survival of the individuals: neurotransmission, peroxisome proliferation/oxidative stress, detoxification potential and anaerobic metabolism, respectively. Additionally, the MN test, the ENA assay and the histopathological analysis of liver from exposed *C. auratus* were also performed.

2. MATERIAL AND METHODS

2.1. Study site

The Cunha Baixa uranium mine (Mangualde, Centre of Portugal), is included in the central uraniferous belt of the Iberian Peninsula (Santos Oliveira and Ávila, 1998). Here, the ore was extracted between 1967 and 1993 through underground and open pit mining (Oliveira and Ávila, 2001) and, in the last few years, the mine pit was filled with pore ore and flooded with sulphuric acid for *in situ* leaching of uranium oxides (Carvalho et al., 2007). The extraction of ore left three ponds: i) one upstream yield by surface mining and immediately abandoned (reference pond - Ref) due to the poorness of extracted ore; ii) the mine pit pond (M), in the underground exploration area, filled with the acidic effluent and, iii) a treatment pond (T) that receives the acidic mine effluent which is pumped from the underground mine, and where hydroxide and barium sulphate are added for neutralization and precipitation of uranium and radionuclides. After treatment the effluent is conducted by a channel to a small stream, a tributary of the Castelo River, and later to the Mondego River, one of the main Portuguese rivers.

2.2. Test organism and laboratorial acclimation conditions

The goldfish *Carassius auratus* was chosen as test organism to assess the effectiveness of the chemical treatment, applied to uranium acid mine drainage, in the mitigation of its ecotoxicological impact on local freshwater resources. This fish species shows an unusual high plasticity and resistance, adaptability to subsist in inauspicious ambient conditions, such as contaminated waters, extreme temperatures and low concentration of dissolved oxygen (Oliveira, 2007). Moreover, this species has a large biological representativeness, since it is a secondary consumer in the habitats where they were introduced. As far as food habits are concerned, the goldfish is an omnivorous fish that ingests algae, detritus, benthic diatoms and filamentous (Xie et al., 2005). Previous studies have shown that goldfish are widely used as model organisms in physiological research (Luo et al., 2006) and commonly used to evaluate the quality of aquatic environments (Sun et al., 2006).

Animals with 7-9 cm body length and approximately 10 g body weight were purchased from a commercial supplier. The first phase of laboratory maintenance involved a period of quarantine (14 days), in which the animals were kept in synthetic hard water medium (ASTM, 1980), and acclimated to continuous aeration, at a temperature of 20±1ºC and a photoperiod of $16h^{L}$: $8h^{D}$. Inspections were conducted twice a day in order to discard wounded, diseased and dead animals. Animals were fed every other day with

an *ad libitum* ration of standard flake food (SERA VIPAN®) and the medium was changed each 2/3 days.

2.3. Test chambers and in situ assays

After the acclimation period, animals were transported to the mine, in synthetic hard water medium (ASTM, 1980) for a maximum travel duration of 1h. Test chambers were made of 5L polystyrene water bottles, thoroughly rinsed with distilled water. In order to allow the entrance of the water/effluent from the ponds into the chambers, in the middle of each side of the bottles a squared area of plastic was cut and removed. The holes were covered with 300mm nylon mesh, using white thermal glue (supplied by Elis-Taiwan, Taiwan, ref. TN122/WS, with a chemical composition of 50% ethylene-vinyl-acetate copolymer, 45% synthetic hydrocarbon, and 5% polyethylene wax), which has been shown to be non-toxic to freshwater organisms (Pereira et al., 2000). Moreover, flotation devices were fixed to the chambers to prevent sinking.

Twelve test chambers, each one with 3 fishes, were exposed within the Ref, M and the T ponds of the Cunha Baixa uranium mine area, for different exposure periods (8, 16, 24 and 48 hours). In each exposure period three chambers were removed from each pond, in a total amount of 9 fish per pond. No food was provided during the tests. Parameters such as pH, temperature, dissolved oxygen concentration, hardness and conductivity were monitored at 8, 16, 24 and 48h, for test validation purposes. Nevertheless, since little variation was observed only values recorded at the end of the test are reported. A total of 108 fish were exposed *in situ* within the three mine ponds (Ref, T and M). At the end of each exposure period, animals were sacrificed on ice-cold phosphate buffer, and total head, liver and dorsal muscle were separated for the development of biomarker assays. At this moment, samples were frozen in liquid nitrogen (in the field), to be transported to the laboratory and stored at -80ºC. Blood was immediately collected to the surface of glass slides to prepare blood smears for the evaluation of erythrocytic nuclear abnormalities (see section 2.6.). In our study, *in situ* tests were conducted in the Ref, T and M ponds, but in the effluent from the M pond fishes died after an 8h-period. However, the acute toxicity of this effluent was already confirmed for a battery of freshwater species (Antunes et al. 2007a, 2007b), as well for fish species (unpublished data).

2.4. Physical and chemical characterization of pond water samples

Water samples were collected from each pond with 500 ml polystyrene water bottles, which were completely filled before closing the lid, transported to the laboratory (maximum travel duration: 1h) and stored at 4ºC, until analyses were performed (maximum storage time: one week). Samples for total metal content analysis were acidified *in situ* with *pro analysis* nitric acid 65% (v/v) MERCK®, to pH<2 (to reduce adsorption phenomena) and were stored in a plastic bottle, at 4ºC, until determinations were possible.

In the laboratory, water samples from both ponds were analyzed for selected metals through ICP-MS spectrometry (APHA, 1995). Additionally, hardness (murexide method – Lange and Vejdelek, 1980) was quantified in aliquots of each sample.

General physical-chemical parameters were measured and recorded both at the beginning and at the end of the *in situ* bioassay. Conductivity was measured with a LF 330/SET conductivity meter; pH measurements were performed using a pre-calibrated WTW 330 SET-2 pH meter and dissolved oxygen concentrations were determined with a WTW OXI 320 oxygen meter. A min/max thermometer was used to measure water temperature.

2.5. Enzymatic biomarker assays

For the development of biomarker assays, samples were withdrawn from the above-mentioned temperature and thawed on ice. Liver tissue was homogenized in ice-cold phosphate buffer (50 mM, pH=7.0 with 0.1% Triton), total head was homogenized in ice-cold phosphate buffer (0.1 M, pH=7.2), and dorsal muscle was homogenized in ice-cold TRIS buffer (0.1 M, pH=7.2). Tissues/organs were homogenized with a tissue homogeniser (YSTRAL D-79282), at a temperature of 4ºC. For the determination of LDH, samples were frozen a thawed three consecutive times, to allow the rupture of the cellular membrane of muscle cells, in order to liberate the intracellular pool of enzyme present.

After homogenization, samples were centrifuged using a refrigerated centrifuge (4ºC). AChE, LDH and GSTs determinations were performed using a spectrophotometer with a microplate reader (Labsystems®, model Multiskan Ex). CAT determinations were performed using a spectrophotometer JENWAY, model 6405 UV/VIS.

Previous work by Yi et al. (2006) showed that the predominant cholinesterasic form present in *C. auratus* brain is acetylcholinesterase. AChE activity was determined in total head homogenates by the method of Ellman et al. (1961). This method is based on

the degradation of acethylthiocholine (structural analogue of acetylcholine) by acetylcholinesterase present in samples, with the subsequent formation of thiocoline. This compound forms a complex with 5,5`-dithio-bis-nitrobenzoic acid (DTNB), allowing the possibility of spectrophotometrical measurement at a wavelength of 412 nm. Results are expressed as nmol/min/mg protein.

CAT was determined in liver homogenates by the method described by Aebi (1984). The velocity of enzymatic decomposition of hydrogen peroxide is proportional to the amount of peroxide present in the reaction medium. Thus, it is possible to quantify the amount of active enzyme in the cellular suspension following the decrease of absorbance at 240nm, which is proportional to the decrease of the substrate (hydrogen peroxide) concentration. Results are expressed as enzymatic activity per minute, per milligram of protein.

GSTs activity was determined in liver homogenates according to Habig et al. (1974). GSTs catalyse the conjugation of the substrate chloro-dinitrobenzene (CDNB) with gluthatione, forming a thioeter, whose formation can be spectrophotometrically followed by the increment of absorbance at a wavelength of 340 nm. Results were expressed as nmol/min/mg protein.

LDH activity was determined in dorsal muscle homogenates following the method of Vassault (1983) adapted to microplate (Diamantino et al., 2001). It consists in piruvate (substrate) reduction and simultaneous β-NADH oxidation by the activity of lactate dehydrogenase present in the samples. β-NADH oxidation can be spectrophotometrically followed by the decrease of absorbance at a wavelength of 340 nm. Results were expressed as mmol/min/mg protein.

Protein quantification of the biological samples was performed using the Bradford method (Bradford, 1976), adapted to a microplate reader, in order to express enzymatic activities per milligram of protein.

2.6. Genotoxic damage and immature erythrocytic frequency

In order to evaluate genotoxic effects, the MN test and the ENA assay were performed in mature peripheral erythrocytes (Carrasco et al., 1990). Immature erythrocytes (IE) frequency was also estimated in order to assess alterations on the haematological dynamics. For such purposes three blood smears were performed per animal. After fixation in pure methanol for 10 min, blood smears were dried (at room temperature) and stained with Giemsa (5%) solution for 30 min. For each slide, 1000

erytrocytes were scored under a 1000x magnification, using an Olympus BX41 microscope. According to the classification of Carrasco et al. (1990) the observed nuclear alterations were divided in the following categories: kidney shaped, notched, lobeb and micronucleus. The criteria used for scoring micronuclei, through comparison with the main nucleus, were i) particles with colour and structure similar to those of chromatin, without shining or refraction, ii) size between 1/5 and 1/100 of the main nucleus, without a bridge linking them, and iii) high proximity to the main nucleus without touching it (Minissi et al., 1996).

Additionally, IE frequency was calculated for the total of cells observed per slide, using the equation:

$$\text{IE frequency } (^{0}/_{00}) = (IE/(ME+IE)) \text{ X } 1000$$

IE – immature erythrocytes; ME – mature erythrocytes

2.7. Tissues preparation for light microscopy

Small sections of liver were collected and fixed for 24h in Bouin's fluid. Sequentially dehydration in graded concentrations of ethanol and sample inclusion in paraffin wax was performed. Sections (5-7 μm thick) were obtained and stained with hematoxylin-eosin for light microscopy examination. Observations were made and photographs taken using an Olympus BX41 microscope equipped with an automatic photomicrographic system.

2.8. Statistical analysis

Two-way analyses of variance were performed for each biochemical parameter to test for significant differences between ponds (Ref and T) and among different exposure periods (8, 16, 24 and 48h), as well as for a significant interaction between both factors. When no significant interactions were recorded, but significant differences were performed between exposure periods, a Tukey multiple comparison test was done to perceive which exposure periods were different from each other ($p<0.05$). On the other hand, if significant interactions between pond and exposure period factors were recorded, one-way analyses of variance were computed to compare ponds, for each exposure period independently.

3. RESULTS

3.1. Physical and chemical analyses of pond water samples

Table 1 describes physical and chemical parameters and total metal concentrations recorded in samples from the two studied ponds (Ref and T), as well as maximum recommended values (MRVs) and maximum admissible values (MAVs) of the same parameters, established for water of human consumption by Portuguese legislation in force (MA, 1998). In the pond T, pH level was close to neutrality, while in the Ref pond the pH was highly alkaline (pH=9.51), being well above the MRV by Portuguese legislation.

Table 1 – Physical and chemical parameters and total metal concentrations recorded in the Ref and T ponds. MRV and MVA stand for Maximum Recommendable Values and Maximum Admissible Values of waters for human consumption (MA, 1998). NA stands for not available values.

	Ref	T	MRV	MAV
Hardness $CaCO_3$ (mg/l)	15	840	NA	500
Dissolved O_2 (mg/l)	9.82	6.77	NA	NA
Conductivity (µS/cm)	178	313	400	NA
pH	9.51	7.02	6.5 - 8.4	NA
Temperature (ºC)	23.9	24.8	NA	NA
Metal (µg/l)				
U	12.8	1380	NA	NA
Cd	< 0.1	1.04	NA	NA
Zn	< 10	86	5	NA
Na	8900	17400	NA	NA
Mn	32.4	6340	20	-
Co	0.44	48.3	NA	NA
Mg	1720	56000	NA	NA
Be	<1	1.96	NA	NA
Al	22.3	136	50	200
Ni	< 1	103	NA	50
Pb	<1	<1	NA	50
Sr	13.5	365	NA	NA
V	0.27	0.54	NA	NA
Fe	203	196	50	200

Hardness was extremely high in pond T, being well above the MAV, as well. Conductivity in the T pond was slightly higher than in Ref pond, nevertheless the levels recorded on both ponds were very low and below MRV, as it was expected, at least for the T pond, due to precipitation of ionic complexes. Dissolved oxygen was higher in the Ref pond. Both parameters remained practically unchanged during the *in situ* exposures.

Regarding metal concentrations, higher values were recorded in the T pond for all the elements analyzed, except for Fe, which showed slightly higher concentration in the Ref pond (203 µg/l) and for Pb, which was below detection limit on both sample waters. In the T pond, Mn (6340 µg/l), zinc (86 µg/l) and nickel (103 µg/l) concentrations clearly surpassed the corresponding Portuguese water quality criteria. The same trend was observed for Mn and Fe concentrations, in the Ref pond, but concentrations of both elements were remarkable lower (Mn: 32.4 µg/l; Fe: 203 µg/l). With respect to U, Be and Sr, three of the main concerning elements in the area, no water quality criteria are available in Portuguese legislation.

Nevertheless, the total U concentration recorded in the T pond was well below the predictive no-effect concentration value for freshwater fishes, in hard water (23 mg U/L) determined by Sheppard et al. (2005). However when comparing the concentrations of the three elements with USEPA – surface water screening benchmarks[1] (Be: 5.3 µg/l; Sr: 1500 µg/l; U: 700 µg/l), only uranium surpassed this benchmark value in the T pond.

3.2. Biomarker assays

3.2.1. Oxidative stress biomarkers

Figure 1 shows the variation in AChE activity in fish exposed *in situ* in both mine ponds. No significant differences were recorded for this biochemical parameter among exposure periods ($F=1.176$; $d.f.= 3$; $p=0.350$) and between ponds ($F=4.542$; $d.f.=1$; $p=0.050$).

[1] SW – EPA R6 FW Surface water screening benchmark. Available on http://rais.ornl.gov/cgibin/eco/bench_select (05/05/2008).

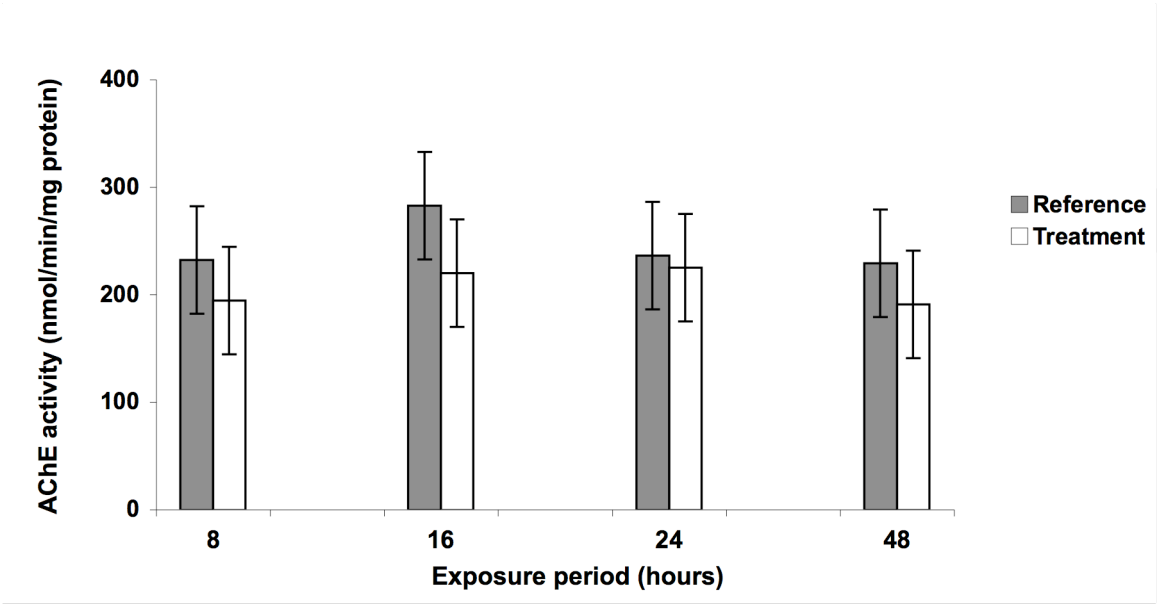

Figure 1 - Variations in AChE activity (expressed as nmol/min/mg protein) in the total head of *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).

The same trend was recorded for CAT (Fig.2), in which no significant differences were recorded as well, when both factors were considered (exposure period: F=0.995, d.f.=3, p=0.420; pond: F=4.465, d.f.=1; p=0.051).

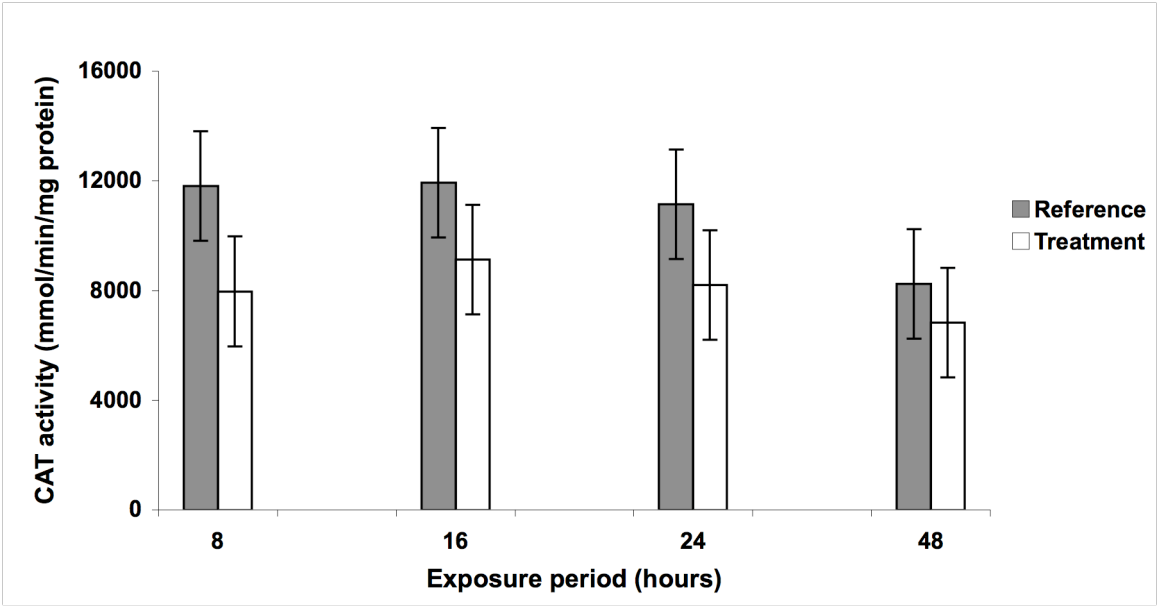

Figure 2 - Variations in CAT activity (expressed as mmol/min/mg protein) in the liver of *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).

The assessment of GSTs activity (Fig.3) showed no significant differences were recorded between exposure periods (F=0.761, d.f.=3, p=0.761). However, significant differences were recorded among ponds (F=22.455, d.f.=1, p=0.001), being this parameter depressed in fish exposed in the T pond. Since a non-significant interaction was recorded between both factors, a Tukey multiple comparison test was performed to discriminate for significant differences among exposure periods. Although the activity of this enzyme was always remarkably higher in the liver of fish exposed in the T pond, significant differences between ponds ($p<0.05$) were recorded only after 16h and 24h of exposure.

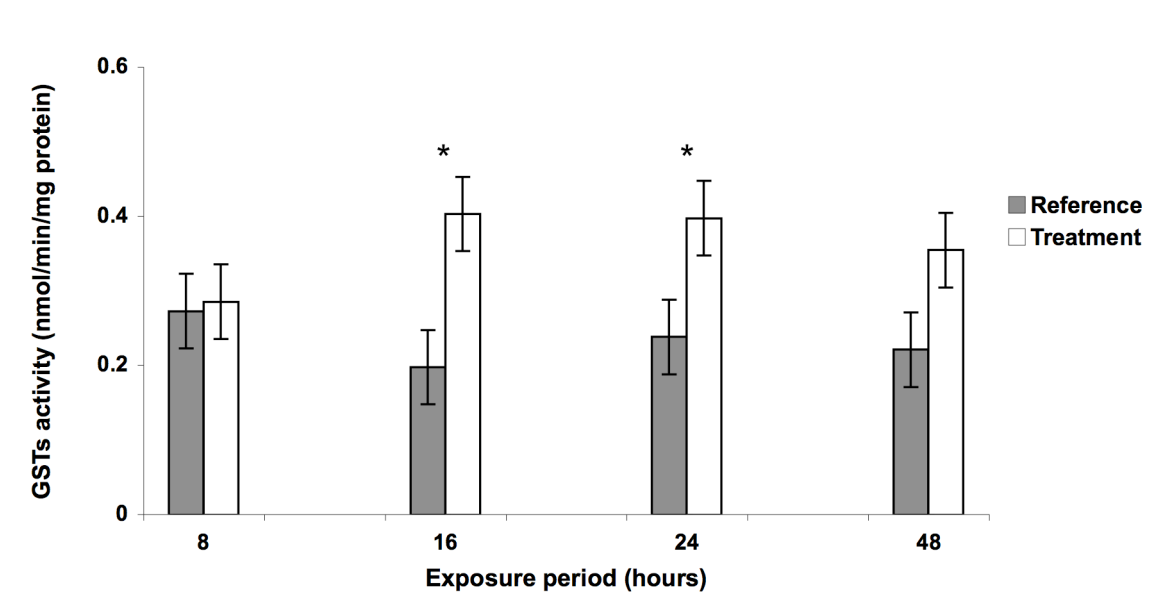

Figure 3 - Variations in GSTs activity (expressed as nmol/min/mg protein) in the liver of *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).
(*) – stands for significant differences among ponds.

For LDH activity (Fig. 4) significant differences between exposure periods (F=3.786, d.f.=3, p=0.032) and ponds (F=20.095, d.f.=1, p<0.001) were recorded. Inasmuch as the interaction between both factors was also significant (F=3.461, d.f.=1, p=0.041), one-way ANOVAS were performed to search for significant differences among ponds at each exposure periods. This parameter was always depressed in fish exposed within the T pond, nevertheless significant differences among ponds were registered only after 8h (F= 12.959, d.f.=1, p=0.023) and 48h (F=53.772, d.f.=1, p=0.002) of exposure.

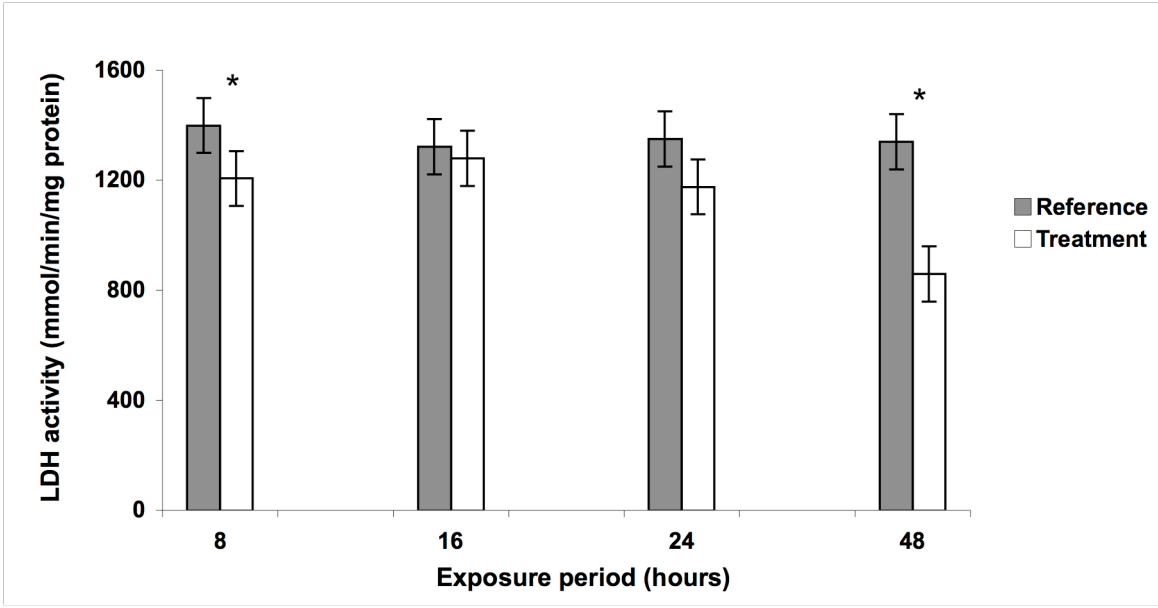

Figure 4 - Variations in LDH activity in the muscle (expressed as mmol/min/mg protein) of *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV). (*) – stands for significant differences among ponds.

3.2.2. Genotoxicity biomarkers

Figure 5 describes MN occurrence in erythrocytes from fish exposed in both ponds for different exposure periods. The two-way ANOVA detected no statistically significant differences both between exposure periods (F=0.280; d.f.=3, 43; p>0.05) and between ponds (F=0.116; d.f.=3, 43; p>0.05).

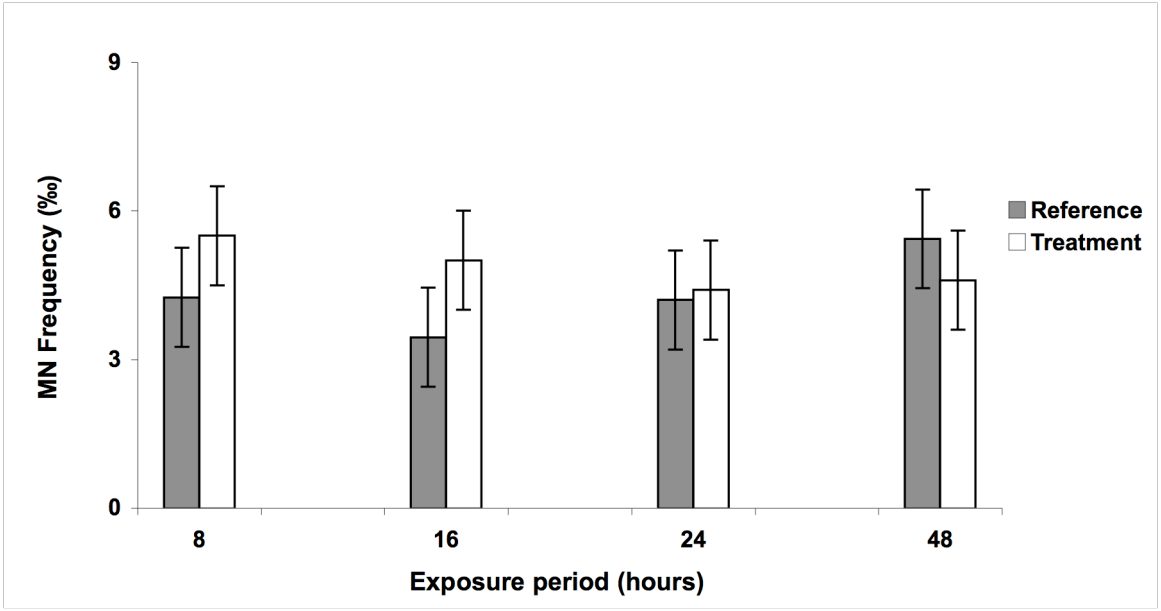

Figure 5 - Variations in the average frequency (‰) of erythrocyte with micronucleus observed in *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).

When the total number of ENA - kidney shaped, notched, lobeb and micronucleus- was considered (Fig.6), no significant differences were recorded among ponds (F=0.148, d.f.=1, p=0.282) but only among exposure periods (F=3.128, d.f.=3, p=0.030). Although, a remarkable increasing pattern was recorded in ENA of fish exposed in both ponds. Notwithstanding, according to the Tukey multiple comparison test performed, significant differences ($p<0.05$) were only recorded in the Ref pond, when comparing exposure periods. Thus, the average number of ENA recorded after 48h of exposure in fish exposed within this pond was significantly higher from levels recorded after 8 and 24h of exposure.

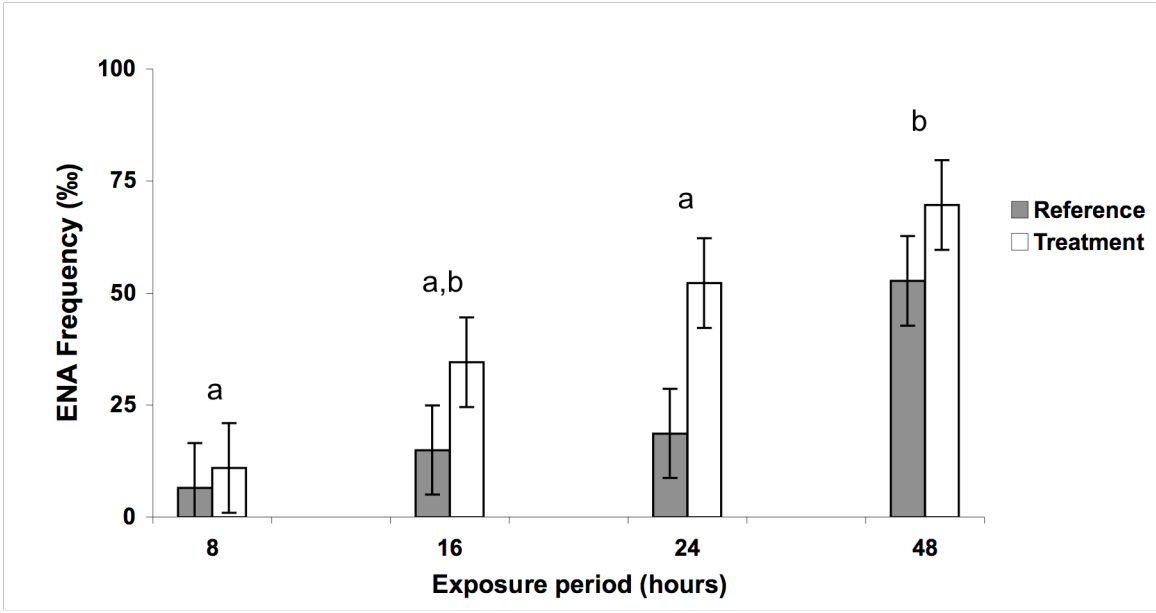

Figure 6 – Variation in the average frequency (‰) of erythrocytes with nuclear abnormalities observed in *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).
a, b – stands for significant differences among periods.

When considering IE frequency (Fig. 7), no significant differences were recorded among ponds (F=1.781; d.f. =1; p=0.189). Once more, this parameter varied significantly among exposure periods (F=21.334; d.f.=3; $p<0.001$). Since no significant interaction was recorded among tested factors, a Tukey multiple comparison test was performed revealing significant differences among IE average values recorded for all the exposure periods ($p<0.05$), except between 16 and 24h of exposure (p=0.313).

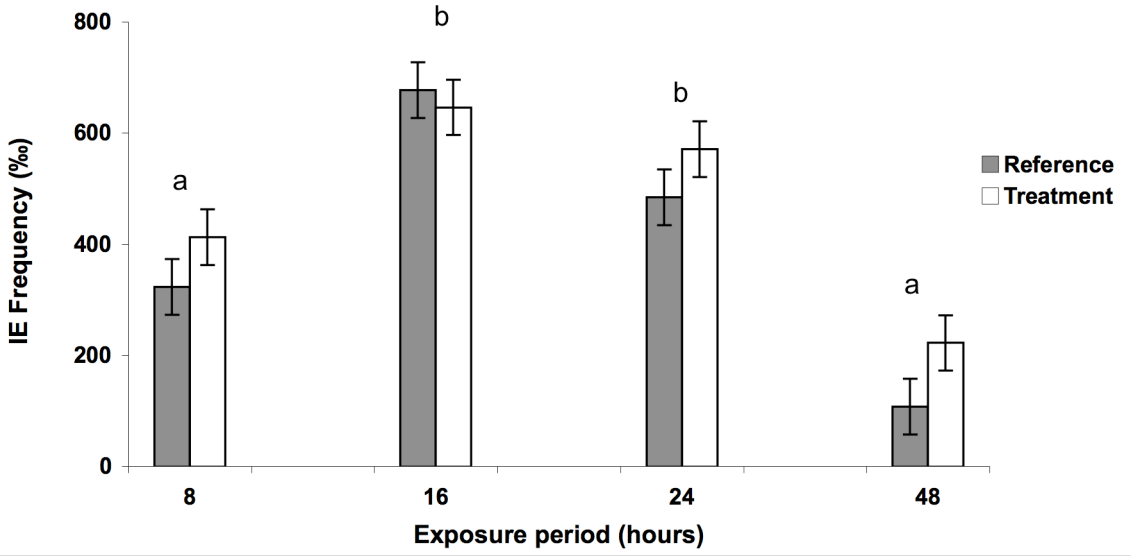

Figure 7 – Variation in immature erythrocyte frequency (‰) observed in of *C. auratus* exposed to both the Ref and the T ponds for different periods (average ± STDEV).
a,b – stands for significant differences among periods.

3.2.3. Histopathological analysis

Animals exposed in both ponds, for different periods, did not show any gross pathology on the liver (figures not shown). In general, the livers of all the animals revealed no morphological and/or structural alterations.

4. DISCUSSION

Remediation measures undertaken in mining areas, after ore extraction activity has stopped, are of paramount importance to mitigate the contamination of surface waters and aquifers. These initiatives are also of importance to reduce the aesthetic damage of the disturbed area and to replace soil topography, as well as surface soil productivity (Ávila et al., 2005; Benedetto et al., 2005; Loredo al., 2003). With respect to mining effluents, in particular, which can have severe toxicological impacts in surface waters, with subsequent loss of sensitive species, biodiversity and ecosystem integrity (Starnes and Gasper, 1995), Portuguese legislation in course obliges the mining companies [Law by Decree nº88/90, from 16th of March, article nº54 (CM, 1990)] to the treatment of wastewaters, to the reestablishment of their quality and their supply to human populations. The observance of such obligations is usually assessed only by comparing main physical

and chemical parameters, as well as metal concentrations in treated effluents, with water quality criteria, before their release into receiving freshwater systems. On one hand, in the national context, this evaluation is overprotective, due to the lack of water quality criteria specifically derived for freshwater organisms, but on the other hand it ignores the combined effects of the mixtures of contaminants present in the effluents. In fact, this chemical evaluation is never strengthened by an ecotoxicological evaluation of the effluent, after chemical treatment, in order to predict the real biological impact of consented discharges.

The results of our study have demonstrated the effectiveness of the chemical treatment applied to the Cunha Baixa uranium mine effluent, in terms of pH neutralization. In fact, low pH can be one of the main stressors of mine effluents affecting freshwater species directly through disturbances on their ionic balance with subsequent effects on survival, growth rates, reproduction (due to endocrine disruption), length of development stages, frequency of molting and migratory behaviors (Chen and Chen, 2003; Vidal et al., 2002; Magee et al., 2001; Ogawa et al., 2001; Horne and Dunson, 1995). Moreover, low pH can also have indirect effects increasing the bioavailability of metals and their ability to yield toxic effects (Gimbert et al., 2008; Luo et al., 2008; Pyle et al., 2002; Oliveira, 1997).

A remarkable reduction in total metal contents of the mine effluent was promoted by the chemical treatment, as can be noticed by comparing the effluent from the T pond, with the effluent from the M pond (Antunes et al., 2007a, b) and by Marques et al. (*in press*). Nevertheless, metals such as Zn, Mn and Ni still showed high concentrations in the chemically treated effluent, quite above those recorded in the Ref pond, and water quality criteria as well. Moreover U and Sr also remain at high concentrations. As far as U is concerned, in spite of the nature of its chemistry, the complexation with carbonate ions, in combination with a pH of 6 to 7, can increase uranium ion solubility, and subsequently their bioavailability to yield toxic effects (Kuhne et al., 2002). Moreover, Franklin et al. (2000) also mentioned the apparent protective effect of H^+ ions (for pH values between 5.7 to 6.5), which can, to a certain level, compete for binding sites in cell surfaces, preventing free metal ions to bind. This H^+ protection may be reduced with the neutralization treatment, leading to increased adsorption of U and other metals to the cells surface, with greater uptake and toxicity. Because of these related factors, observations showed that the chemical treatment that is being applied in the Cunha Baixa uranium mine is less effective in terms of metal contents reduction, giving rise to concerns about it's contribution for the input of toxic concentrations of metals to freshwater nearby resources. As it was explained, this can be difficult to solve with the non-specific treatment applied due to complex mixture of metals present, whose speciation can be differently

affected by water quality variables such as pH, hardness and dissolved organic carbon (Franklin et al., 2000).

Enzymatic biomarkers were chosen as endpoints to assess the effluent impacts on the fish species *Carassius auratus*, exposed *in situ* to the treated uranium mine effluent, because they have been reported as more sensitive endpoints, than those at higher levels of biological organization, and they are usually the first affected by environmental changes (Stegeman, 1992). Comparisons were made with fish exposed in a nearby Reference pond (Ref.), in order to mitigate the influence of some environmental factors such as temperature, oxygen, handling and confinement stress in the interpretation of biomarker responses (Vidal et al., 2002). Nevertheless, Vidal et al. (2002), have also observed the effect of alkaline pH, in the reduction of the activity of antioxidant enzymes such as CAT and NADH-reductase. Therefore, the highly alkaline pH value recorded in the Ref pond, which is determined by the geological properties of pond bottom and the low level of dissolved oxygen recorded in the T pond, may have contributed for the reduction of the activity of some enzymes, mitigating the differences among fish exposed in both ponds.

Heavy metals have been considered as part of the compounds responsible for AChE inhibition (Frasco et al., 2005; Lionetto et al. 2003). In the present study, cholinergic nervous function of *C. auratus* did not seem to be significantly altered following the exposure to the treated effluent, as can be concluded after comparing the average activity of this enzyme with the same activity of fish exposed in the Ref pond. The inhibitory effect of heavy metals which has been observed in specific brain AChE activity may be due to a direct effect, derived from their ability to bind covalently to serine at the active AChE site and/or can be interpreted as a secondary outcome of conformational changes in the enzyme, due to metals binding with functional sulfidryl groups (de la Torre et al., 2007; Gaitonde et al., 2006; Lionetto et al., 2003; Matozzo et al., 2005). Hence, in our study, the high concentrations of metals (such as Zn, Mn, Ni, U and Sr) in the treated effluent from the T pond (Table 1), may explain the slight inhibition of AChE activity in *C. auratus* for all the exposure periods.

Metals are also known by their ability to enhance the formation of reactive oxygen species (ROS; Sun et al., 2006). Dautremepuits et al. (2002), in particular, reported that metal accumulation causes an increase in ROS such as hydrogen peroxide, superoxide radical and hydroxyl radical, leading to oxidative stress in fish. In response of oxygen toxicity that occurs in circumstances associated with pollutant exposure, CAT, an antioxidant enzyme, is used as a marker involved in the primary defense against oxidative damage (Atli et al., 2006; Lionetto et al., 2003; Ahmad et al., 2000). CAT is responsible for the removal of hydrogen peroxide (H_2O_2), produced from the metabolism of long chain

fatty acids in peroxissomes, which is metabolized to molecular oxygen and water. In this way, CAT activity could be induced in response to ROS production, since this enzyme tends to inhibit oxyradical formation. Although, various responses of this enzyme have been observed in animals exposed to metallic contaminants in both field and laboratory experiments, which indicated an increase or a decrease in the activity depending on dose, species and route of exposure (Sanchez et al., 2005; Won and Won, 2000). Our results showed a somewhat mild decrease in CAT activity, according to a time-dependent pattern, on both ponds, more evident after 16h of exposure, suggesting that stress agents other than metals may have influenced this biomarker on the Ref pond (e.g. alkaline pH). Lopes et al. (2001) observed that enzymatic activities may change due to other environmental factors. On the other hand, the decreased levels of CAT activity may be related to direct binding to –SH groups on the enzyme molecules, as it were reported by Atli and Canli (2007). Notwithstanding, in spite of no statistical significant differences among ponds, CAT activity was always lower in fish from the T pond.

Phase II biotransformation enzymes can play an important role in homeostasis as well as in detoxification and clearance of many xenobiotics compounds (Van der Oost et al., 2003). Specifically, the phase II enzymes glutathione-S-transferases (GSTs) are involved in the biotransformation and detoxification of a number of electropholic compounds, by conjugation of the xenobiotic parent compounds, or its metabolites, with glutathione. These conjugations are understood as addition reactions in which polar chemical groups or compounds (such as amino acids, sugars, sulphate, glucuronic acid, or, most important, the tripeptide glutathione) are covalently added to xenobiotic compounds. In this way, the toxicity of many exogenous compounds seems to be modulated via induction of GSTs, as many recent studies have been reported in freshwater fish (Yi et al., 2007; Monteiro et al., 2006; Rao, 2006). GSTs also perform a role in physiologic oxidative stress (Yin et al., 2007; Ozmen et al., 2006; Dautremepuits et al., 2002). In this way, GSTs catalyzes the conjugation of glutathione (GSH) with various electrophilic substances, and play a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Barata et al., 2005). The molecules involved in mechanisms of protection from oxidative stress are correlated with the increase in GSTs activity (Freitas et al., 2007). In our study, fish exposed to the treated effluent had their liver GSTs activity significantly increased, for exposure periods equal to both 16h and 24h. This pattern remained after 48h of exposure; nevertheless differences among ponds were not significant. Since GSTs are involved in both detoxification and antioxidant defense processes, our findings suggest an interference of effluent contaminants (e.g. metals or radionuclides) with at least one of the processes. Since

metals are known to cause oxidative stress in several species (Elumalai et al., 2007; Barata et al., 2005; Zhang et al., 2005; Lopes et al., 2001), it can be hypothesized that the increase of GSTs activity, observed in *C. auratus* exposed to a complex mixture of metals (in T pond), may indicate an oxidative stress response, that promotes a stimulation of oxidative defense mechanisms. These findings are in good agreement with previous studies reporting the role of metals in increasing GSTs activity in fish (Liu et al., 2006; Lopes et al., 2001; Guosheng et al., 1998). However, and in opposition to our results, a significant decrease was described in hepatopancreatic GSTs activity on females of the marine crab *Carcinus maenas* after exposure to Cu and Cr and as well as to mixtures of both metals (Elumalai et al., 2002), and no effects on liver GSTs were found in the fish *P. reticulata* and *G. holbrooki* after an *in situ* exposure to an acid mine drainage containing a complex mixture of metals (Castro et al., 2004). These apparently paradoxical results may be due to species-specific responses of the enzymes at high and low concentrations of xenobiotics. According to Van der Oost et al. (2003), few studies reported GSTs activities to be significantly increased, but in most cases no significant differences were observed between fish from the control and polluted sites. Our results suggest that the hepatic GTSs constitute a sensitive biochemical indicator of oxidative stress promoted by the uranium mine treated effluent in *Carassius auratus*.

LDH is a cytoplasmatic enzyme that may be found in different isoforms, which catalyze the interconversion of pyruvate and lactate in the glycolysis process (Elumalai et al., 2007). Changes in enzymatic activities of LDH reflect disturbances in metabolic pathways and in the cellular capacity to deal with oxidative processes during metabolism (Michaelidis et al., 2007). Several authors have reported both the increase (Elumalai et al., 2007; Castro et al., 2004; Almeida et al., 2001) and the inhibition (Ozmen et al., 2006; Castro et al., 2004; Diamantino et al., 2001) of LDH activity on the muscle and liver of fish and crustacean species, exposed to sublethal concentrations of metals. The dorsal muscle anaerobic capacity, measured by LDH activity, was significantly inhibited in fish exposed to the effluent from the T pond. Nevertheless, significant differences between ponds were recorded after 8 and 48h of exposure. These results suggest a moderate increment of the anaerobic metabolism in the muscle tissue of fish exposed to the treated effluent, probably to compensate for disturbances in the aerobic capacities. Nevertheless, anaerobic capacities also became significantly inhibited after 48h of exposure in the T pond, in comparison with fish from the Ref pond. Hence we can hypothesize the presence of some metals, or other chemical elements not analyzed (e.g. radionuclides) in the treated effluent, which are able to inhibit LDH activity. This was, at least in part, in agreement with Ozmen et al. (2006), which observed a good correlation between

decreased liver LDH activity in *Cyprinus carpio*, captured in the Karakaya Dam Lake (in Turkey), with metal (e.g. Cd, Cu and Pb) concentrations recorded.

The relationship between erythrocyte MN frequency in peripheral blood and aquatic pollution levels has been observed in freshwater fish (Barni et al., 2007; Çavaş and Ergene-Gözükara, 2003; Minissi et al., 1996). In particular, Norppa and Falck (2003) reported that the increase of micronucleated erythrocytes indicates that pollutants may induce genotoxic effects on proliferating stem cells. In the present study, the average frequency of MN recorded in fish exposed in the T pond was not significantly different from the frequencies recorded in fish exposed in the Ref pond, for all the exposure periods.

Although the mechanisms underlying the formation of ENA have not been fully explained, these abnormalities are considered to be indicators of genotoxic surveys (Çavaş and Ergene-Gözükara, 2005; Fenech and Crott, 2002; Serrano-Garcia and Montero-Montoya, 2001). Our results of the ENA assay showed a significant increase of total nuclear abnormalities, albeit without significant differences between ponds. This increasing trend with the exposure duration may have been enhanced by the significant reduction in blood cells renewal, as can be confirmed by the low IE frequency, recorded on both ponds. In fact, a recent study (Udroiu, 2006) showed that the appearance of nuclear lesions is widely affected by a variety of factors such as erythropoiesis, time required for maturation and the lifespan of the erythrocytes. In our study, the existence of a similar pattern between ponds, suggests that factors other than metals, may have been responsible either by an increase in occurrence of erythrocyte nuclear lesions or by a lower rate of renovation of blood cells, with a subsequent increase in mature cells with nuclear alterations occurring spontaneously.

In spite of the recorded enzymatic alterations, indicating that organisms were exposed to oxidative stress agents, the liver of fish exposed in both ponds did not reveal significant morphological changes in their structure. Since histophatological changes were already recorded on fish exposed to metals, uranium included (e.g. Cooley et al., 2002; Cooley et al., 2000), we believe that these results are mainly explained by the reduced timeframe of the assay. Reduced exposure periods are an important requisite of *in situ* bioassays, to reduce confinement stress of organisms, costs, time required to obtain results and to prevent vandalism actions. Such endpoints, such as tissue alterations, seemed not to be appropriate for such timeframes, since they are much more likely to occur following long-term exposures.

5. CONCLUSION

The present study was performed *in situ*, within mine ponds, to record responses of sub-lethal enzymatic endpoints, in real environmental scenarios, under the influence of natural environmental conditions. This was the first study addressing the effectiveness of a chemical treatment widely applied to uranium mine effluents to mitigate their impacts in freshwater environments. It was demonstrated that, although the indisputable effectiveness of the treatment in terms of pH neutralization, some metals still persist at high concentrations; the concentrations in which they are present may be sufficient to induce oxidative stress in fish. In general terms, oxidative stress/respiratory enzymes have proved to be sensitive endpoints for appropriate *in situ* exposure timeframes, in opposition to liver histopathological alterations. Nevertheless, a battery of enzymatic/histological/genetic damage endpoints should always be assessed, at different moments, since their responses are likely influenced by variables other than contaminants, and showed some variation with exposure durations. As it was demonstrated, we can only conclude that organisms are under oxidative stress, with a weight-of-evidence analysis based on the responses of different biomarkers.

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CAPÍTULO III

Conclusões Gerais

Conclusões Gerais

O incremento da poluição ambiental decorrente das actividades humanas exige o desenvolvimento de novas metodologias, tanto para avaliação, compreensão, prevenção e remediação dos danos gerados, quanto para monitorização das acções antropogénicas exercidas que contribuem para a degradação do ecossistemas. Análises físicas e químicas permitem a identificação e a quantificação de poluentes. Assim, para uma efectiva compreensão dos efeitos dos poluentes na componente biológica, incluem-se métodos que evidenciam as consequências directas para os organismos vivos – os bioensaios. Vários são os parâmetros biológicos que podem ser alterados como consequência da interacção entre o agente químico e o organismo. No entanto, a determinação quantitativa desses parâmetros usados como indicadores biológicos ou biomarcadores, só é possível se existir correlação com a intensidade da exposição e/ou o efeito biológico da substância. Desta forma, a utilização de respostas induzidas por xenobióticos em componentes moleculares, celulares ou bioquímicos, apresentam um grande potencial como ferramentas de avaliação de sinais precoces de alteração ao nível sub-individual, individual e até, possivelmente, ao nível da população ou da comunidade.

O presente trabalho teve como principal objectivo o estudo integrado de respostas bioquímicas/metabólicas e citogenéticas, assim como o efeito do stresse químico e radioactividade avaliado em função de alterações histopatológicas em tecidos alvo, pretendendo contribuir para um melhor entendimento sobre a interacção toxicológica que se estabelece entre diversas espécies metálicas, baixos valores de pH, radioactividade e os parâmetros biológicos acima referidos, em peixes.

Uma das observações mais importantes reflectiu-se nos efeitos de letalidade aguda que decorreram da exposição de organismos ao efluente ácido da mina (M). De facto, a mistura de diversas espécies metálicas em elevada concentração, juntamente com os efeitos decorrentes da eventual radioactividade resultante da presença de minério de urânio, e um pH extremamente baixo, constituíram uma combinação que se revelou fatal para a totalidade dos organismos expostos ao efluente M, mesmo após um curto período (cerca de 8 horas). Tal facto inviabilizou a análise de tecidos, embora os resultados de letalidade fossem suficientes para concluir acerca da elevada toxicidade apresentada pelo cito efluente.

A análise comparativa das respostas metabólicas nos três tecidos avaliados – fígado, músculo e tecido nervoso – revelou que o músculo dorsal foi o tecido que mais indiciou o potencial toxicológico do efluente de tratamento (T). A resposta da enzima lactato-desidrogenase (LDH) reflectiu a duração da exposição, assim como as

dissemelhanças entre as características químicas dos efluentes em estudo, uma vez que às 48h os peixes expostos ao efluente T apresentam uma diminuição significativa da actividade da LDH, relativamente aos valores observados nos organismos expostos ao efluente de referência (Ref). Assim, a estimulação respiratória do organismo e consequente passagem para um regime predominante de aerobiose (que resulta num maior consumo de oxigénio), levou à diminuição da actividade desta enzima, podendo esta resposta ter-se ficado a dever à exposição química do efluente. Estes resultados vão de encontro a estudos anteriormente realizados em peixes expostos a metais (e.g. Antognelli et al., 2003; Almeida et al., 2001).

O fígado foi o segundo alvo preferencial para a expressão do stresse químico, sobretudo pela indução de enzimas de destoxificação, como é o caso do aumento significativo da actividade das glutationa-S-transferases (GSTs). Os resultados obtidos revelaram o aumento da actividade das GSTs, a partir das 16h, nos organismos expostos ao efluente T. O aumento da actividade deste grupo de enzimas em peixes expostos ao efeito de metais, encontra-se já documentado (e.g. Liu et al., 2006; Lopes et al., 2001; Guosheng et al., 1998) A actividade da catalase (CAT) hepática, de acordo com o aumento do tempo de exposição, demonstrou um sensível decréscimo, embora não significativo, nos peixes expostos ao efluente Ref. Deste modo, o fígado mostrou ser capaz de reflectir a sua capacidade metabólica através do aumento da sua capacidade de conjugação enzimática via glutationa-S-transferases e/ou pela estimulação dos mecanismos de defesa de stresse oxidativo.

No tecido nervoso, os mecanismos de neurotransmissão não evidenciaram alterações significativas, apesar da actividade da acetilcolinesterase (AChE) apresentar um sensível decréscimo nos peixes expostos ao efluente T. Esta inibição pode estar relacionada com o potencial toxicológico das espécies metálicas presentes, facto reportado em investigações recentes (e.g. de la Torre et al., 2007; Frasco et al., 2005; Lionetto et al., 2003).

O teste dos micronúcleos (MN) foi seleccionado para avaliar a genotoxicidade dos metais no sangue, sem contudo se terem observado diferenças significativas nos animais expostos aos efluentes Ref e T. Relativamente à presença de outras anomalias nucleares eritrocíticas (ANE), observou-se um aumento significativo das mesmas ao longo do tempo, mas não entre locais. Assim, este aumento de ANE pode estar relacionado com a redução significativa da taxa de renovação de células sanguíneas, facto que está em conformidade com o simultâneo aumento da frequência de eritrócitos imaturos (IE) no sangue, observado em ambos os efluentes. Por outro lado, a presença de outros

factores para além da presença de metais, poderá explicar as semelhantes frequências de ANE observadas nos efluentes Ref e T, ao longo do tempo.

Os dados histopatológicos não sinalizam evidências de alterações morfológicas significativas ao nível do fígado, em peixes expostos aos dois efluentes em estudo. Esta aparente ausência de resultados talvez se deva aos curtos tempos de exposição seleccionados, visto que a principal limitação deste biomarcador tem a ver com o facto de, em princípio, não ser aplicável a exposições de curto prazo (Pacheco, 1999).

Em Portugal, a legislação vigente da qualidade da água para consumo humano encontra-se bastante deficitária no que respeita aos valores-limite estabelecidos para a concentração de metais e de outros contaminantes, facto que veio a limitar a interpretação dos dados de caracterização química.

Os resultados obtidos neste estudo sugerem que a espécie *Carassius auratus* evidencia uma sensibilidade adequada, sendo um bom organismo-teste para a monitorização da contaminação dos ambientes aquáticos por metais e/ou por misturas complexas de metais. Porém, é pertinente referir que os períodos de exposição seleccionados foram bastante reduzidos para que se pudessem tirar conclusões precisas relativamente às alterações fisiológicas e bioquímicas provocadas pela exposição ao efluente T da mina, contaminado com diversas espécies metálicas, entre as quais o urânio. De acordo com este princípio, o aumento do tempo de exposição deverá ser um critério a adoptar em trabalhos futuros, assim como a inclusão da análise de outros tecidos, tais como as brânquias e o rim, nomeadamente ao nível da acumulação de metais e do estudo integrado de outros biomarcadores de nível bioquímico/metabólico, como por exemplo, a actividade das metalotioneínas (MTs), da etoxiresorufina-*O*-desetilase (EROD), da determinação do conteúdo em P450 e sua degradação, e da peroxidação lipídica (LPO). Concomitantemente, este estudo poderia estender-se a outras espécies de diferentes níveis tróficos, quer aplicando testes *in situ* em organismos, quer testes laboratoriais de modo a validar-se os resultados de campo. A integração destes resultados poderá vir a possibilitar uma melhor compreensão das alterações biológicas supracitadas em organismos seleccionados, com vista a uma avaliação mais abrangente e mais realista dos potenciais impactos decorrentes de efluentes de minas abandonadas no Interior de Portugal.

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